Genetic Relationships Among *Leptographium terebrantis* and the Mycangial Fungi of Three Western *Dendroctonus* Bark Beetles

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Abstract: Morphology, mitochondrial DNA (mtDNA) restriction fragment polymorphisms (RFLPs) and nuclear DNA (nDNA) fingerprinting were used to clarify relationships among the morphologically similar Ophiostoma and Leptographium species associated with mycangia of three Dendroctonus bark beetles (Ophiostoma clavigerum associated with both D. ponderosae and D. jeffreyi, and L. pyrini-um associated with D. adjunctus), as well as a closely related nonmycangial bark beetle associate (L. terebrantis). Most isolates of O. clavigerum form long (40–70 μm), septic conidia, while all isolates of L. terebrantis and L. pyrini-um form conidia less than 17.0 μm in length. The conidia of L. pyrini-um are pyri-form, with truncate bases, while the conidia of the other species form only slightly truncate bases. Conidial masses of L. terebrantis are creamy yellow, while the conidial masses of the other species are white. Nuclear DNA fingerprints resulting from probing PsiI restrictions with the oligonucleotide probe (CAC)₅ and HindIII and MspI restrictions of mtDNA, exhibited three major clusters. In the dendrogram developed from mtDNA RFLPs, the L. pyrini-um isolates formed one cluster, while the majority of O. clavigerum isolates, including all D. jeffreyi isolates, formed another. A third cluster was composed of all L. terebrantis isolates, as well as several O. clavigerum isolates from D. ponderosae. The inclusion of some O. clavigerum isolates in the L. terebrantis cluster suggests that horizontal transfer of mtDNA has occurred among these fungi. The nDNA dendrogram also ex-
hibited three clusters, and most isolates of L. pyrini-um, L. terebrantis and O. clavigerum grouped separately; however, one isolate of O. clavigerum grouped with the L. terebrantis isolates, while one isolate of L. terebrantis grouped with O. clavigerum. No genetic markers were found that distinguished between O. clavigerum associated with D. ponderosae and O. clavigerum associated with D. jeffreyi. Ophiostoma clavigerum might be a recently diverged morphological variant of L. terebrantis, with special adaptations for grazing by young adults of D. jeffreyi and D. ponderosae. The anamorph of O. clavigerum, Graphiocladiella clavigerum, is transferred to Leptographium.

Key words: Dendroctonus adjunctus, D. jeffreyi, D. ponderosae, DNA fingerprinting, Leptographium pyrini-um, L. terebrantis, mitochondrial DNA, mycangial fungi, Ophiostoma clavigerum, RFLP, Scolytidae

INTRODUCTION

Several bark beetles (Scolytidae: Coleoptera) in the genus Dendroctonus Erichson possess mycangia formed by invaginations of the exoskeleton that function in the dissemination of symbiotic fungi. Many associations among Dendroctonus bark beetles and mycangial fungi are believed to be mutualistic due to the presence of these specialized structures, which clearly are involved in the maintenance of the association. These associations appear to be obligate; the fungi are highly adapted to dissemination by insects (Malloch and Blackwell 1993) and apparently are dependent wholly on the host beetle for dispersal. Likewise, the beetles appear to be dependent upon at least some mycangial fungi for successful development and reproduction (Whitney 1971, Barras 1973, Bridges 1983, Goldhammer et al 1990, Coppedge et al 1995, Six and Paine 1998, Ayres et al 2000).

Among the many ascomycetes associated with Den-
droctonus mycangia are several blue-staining fungi in the genus Ophiostoma H. & P. Sydow and the asexual genus Leptographium Lagerb. & Melin. (many species of Ophiostoma possess Leptographium anamorphs) (Harrington 1988). Dendroctonus jeffreyi Hopkins and D. ponderosae Hopkisis carry Ophiostoma clavigerum (Robinson-Jeffrey & Davidson) Harrington in their mycangia (Whitney and Farris 1970, Six and Paine 1997), while D. adjunctus Blandford carries Leptogra-

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<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate No.</th>
<th>Origin</th>
<th>Beetle associate</th>
<th>Tree species</th>
<th>Color of conidial mass</th>
<th>Conidium length (µm)</th>
<th>Number of conidial septa</th>
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<td><em>L. pyrinum</em></td>
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<td>White</td>
<td>120–16.0</td>
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<td>110–16.0</td>
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<td><em>P. laubertiana</em></td>
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<td>38–70</td>
<td>0–3</td>
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<td><em>P. laubertiana</em></td>
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<td>Species</td>
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<td>Beetle associate</td>
<td>Tree species</td>
<td>Color of conidial mass</td>
<td>Conidium length (μm)</td>
<td>Number of conidial septa</td>
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* Isolates beginning with C are from the culture collection of T. C. Harrington. Isolates beginning with DLS are from the culture collection of D. L. Six.
Mycologia

...phium pyrinum Davidson (Six and Paine 1996). The anamorph of Ophiostoma clavigerum and L. pyrinum are similar but morphologically distinct, and the two fungi exhibit high genetic identity with one another based on isozyme markers (Zambino and Harrington 1992, Six and Paine 1999a). Leptographium terebrantis also is similar morphologically to the anamorph of O. clavigerum; it has been isolated from many species of Pinus across North America and from a number of bark beetles, including D. valens LeConte, D. terebrantis (Oliv.) and Hylurgops porosus (LeConte), but it has not been found associated with beetle mycangia (Harrington 1988). Many Ophiostoma and Leptographium species exhibit a high degree of pleomorphism (Malloch and Blackwell 1993), and Tsuneda and Hiratsuka (1984) found a wide range in conidophore and conidial morphology in O. clavigerum. This, and the high degree of similarity in isozyme phenotypes, led Zambino and Harrington (1992) to suggest that O. clavigerum, L. pyrinum, L. terebrantis and some other Leptographium species may be morphological variants of a single species.

Some uncertainty also exists whether O. clavigerum associated with D. jeffreyi and O. clavigerum associated with P. ponderosa are constitute a single species or comprise a pair of cryptic species or physiologic races. No differences were seen in O. clavigerum isolates from the mycangia of the two beetles using morphology, isozymes or temperature tolerances (Six and Paine 1997). This fungus, however, when isolated from the two beetles, exhibits significant differences in tolerances for host tree-resin components, with greater tolerances for host resins than non-host resins when grown in artificial culture (Paine and Hanlon 1994). Ophiostoma clavigerum associated with the two beetles also exhibited differential growth in bolts of Pinus contorta Dougl. and P. jeffreyi Grev. & Balf., hosts of P. ponderosa and D. jeffreyi, respectively (Six and Paine 1998). Dendroctonus jeffreyi and D. ponderosa are sibling species that are morphologically and genetically very similar (Highy and Stock 1982, Wood 1982). However, there is no overlap in host tree species used by the two beetle species. Dendroctonus jeffreyi is monophagous and attacks only P. jeffreyi (Wood 1982). Dendroctonus ponderosa is polyphagous, attacking 13 species of Pinus but not P. jeffreyi (Wood 1982). The major resin components of the host trees of the two beetles differ considerably, n-Heptane is the major resin component of P. jeffreyi, while the main resin components of hosts of D. ponderosa are monoterpenes and resin acids (Mirov 1929, Smith 1967, Anderson et al 1969). Therefore, O. clavigerum associated with D. jeffreyi and O. clavigerum associated with D. ponderosa are isolated in different chemical environments, which ultimately may result in divergence due to selection and random genetic drift. Divergence in morphologically simple fungi may result in genetic differentiation without concurrent morphological changes (Kemp 1977, Brasier 1986).

Our objectives were (1) to assess whether L. pyrinum is a species distinct from O. clavigerum or simply a morphologic variant, (2) to determine whether differentiation is occurring between O. clavigerum associated with D. jeffreyi and O. clavigerum associated with P. ponderosa and (3) to assess the relationship of the nonmycangial L. terebrantis to O. clavigerum and L. pyrinum. In addition to morphological comparisons, we used nuclear DNA (nDNA) fingerprinting and restriction fragment-length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA), techniques that have been used successfully to differentiate among species and strains of several fungi (Meyer et al 1991, DeSzenco and Harrington 1994).

MATERIALS AND METHODS

Isolates.—Isolates used in this study are listed in Table I. Most isolates were obtained directly from mycangia of bark beetles. The procedure used for mycangial isolations is presented elsewhere (Six and Paine 1997). Ophiostoma clavigerum was isolated from D. ponderosa (DP) collected at three locations in California (isolates C836, C837, C838, C839 and C841) where D. ponderosa and D. jeffreyi are sympatric. Ophiostoma clavigerum also was isolated from D. jeffreyi (DJ) collected at 10 California sites that are representative of a majority of the geographic range of that beetle. In most, if not all, of these locations, D. jeffreyi and D. ponderosa are sympatric (Wood 1982). Fungi from allopatric populations of D. ponderosa also were included for comparison (C295, C293 and C86).

Morphology.— Cultures were grown in 95 mm plastic Petri dishes with MYEA (2% malt extract, 0.2% yeast extract and 1.5% agar) and autoclaved Pinus strobis twigs for at least 3 wk. Microscope slides were prepared of conidiphores and conidia produced near the twigs by mounting in cotton blue. For each isolate, 10 of the longest conidia were measured at 500× magnification and the number of septa was noted for each conidium.

Total genomic DNA extraction. Mycelia were grown in 25 mL liquid medium (2% malt extract, 0.2% yeast extract and 1.5% agar) in 125 mL Erlenmeyer flasks and held at room temperature (ca 21 C) in the light. After 14 d the mycelial mats were collected and dried with vacuum filtration through 1 mm Whatman No. 1 filter paper (Whatman International Ltd., Kent, England). The mats were placed between paper towels and dried for an additional 10 min. Immediately after drying, the mats were ground with mortar and pestle to a fine powder in liquid nitrogen.

Total genomic DNA extractions were carried out with the ground mycelia and a modification of the method devel-

mtDNA RFLPs.—RFLPs of mtDNA can be visualized directly from stained gels of total genomic DNA that has been digested with restriction enzymes possessing G-C, four-base recognition sites (Wingfield et al. 1996). mtDNA exhibits a high degree of restriction fragment-length polymorphism at the intraspecific level, and length mutations have been shown to be the major cause of this variation (Sanders et al. 1977, Taylor et al. 1986, Bruns et al. 1988). Total genomic DNA was digested with HaeIII and MspI restriction enzymes (Promega, Madison, Wisconsin), which recognize the base sequences GGCC and CCGG, respectively. These enzymes digest the majority of nuclear DNA to relatively short lengths, leaving relatively long pieces of AT-rich DNA, which are primarily from the mitochondrial genome. After electrophoresis, ethidium bromide-stained bands of uniform intensity are scored as mtDNA bands. Protocols used for electrophoresis and staining of RFLPs were developed by Wingfield et al. (1996). mtDNA RFLPs were not obtained for isolates C186, C847 and DLS568, and nDNA fingerprints were not obtained for isolate 608.

nDNA fingerprinting. nDNA fingerprints are produced by the hybridization of DNA probes (oligonucleotides) to restriction fragments of genomic DNA. These probes are homologous to hypervariable repetitive sequences often called “simple repetitive sequences” or “microsatellite DNAs”. These simple repetitive sequences often exhibit substantial variability in their repeat copy number for a given locus.

For nDNA fingerprinting, total genomic DNA was digested using the restriction enzyme PstI (Promega, Madison, Wisconsin) and then hybridized with the synthetic oligonucleotide probe (CAC). This probe has been shown to be useful in detecting variation in both basidiomycetes and ascomycetes (DeScenzo and Harrington 1994). Protocols for PstI digestion, electrophoresis, radiolabeling, in-gel hybridization and autoradiography are described in DeScenzo and Harrington (1994).
Data analysis.—Band sizes for both mtDNA RFLPs and nDNA fingerprints were determined using Gelreader (version 2.0.5) (National Center for Supercomputing Applications 1991). Bands of the same molecular weight were scored as alleles possessing two character states (presence/absence). Gels used for DNA fingerprinting were run twice, and only bands distinct and scorable in both runs were analyzed. Cluster analysis was performed using the GENDIST (Nei’s genetic distance) and NEIGHBOR (UPGMA, unweighted pair-group method with arithmetic averaging) programs found within the PHYLIP package (version 3.5) (Felsenstein 1993). Trees were produced from PHYLIP files using TREEVIEW (version 1.6.6.) (Page 1996).

RESULTS

Morphology.—Three morphological species were recognized by conidial characteristics (Table I, Figs. 1–16). Conidial masses of L. terebrantis were creamy yellow en masse when examined under a dissecting microscope, while all isolates of L. pyriform and O. clavigerum had white conidal masses. The conidia of L. pyriform were 4–7 μm wide, had strongly flattened bases and tended to be pyriform to obovoid; while conidia of the other species were 2–4 μm wide, had bases that are slightly truncate to rounded, and were cylindrical, to obovoid, to clavate. The length of L. pyriform conidia was found to be 10–17 μm, those of L. terebrantis to be 6–12 μm and those of O. clavigerum to be highly variable. Most isolates of O. clavigerum formed clavate-shaped conidia that were large (40–70 μm in length), with up to four septa, but smaller conidia, similar to those of L. terebrantis in size and shape, were common in all isolates of O. clavigerum. A number of isolates of O. clavigerum produced only small conidia of the L. terebrantis type in the examination reported in Table I but produced larger conidia in earlier examinations. Isolate C813 earlier produced clavate conidia from 10–35 μm in length, and its conidial mass was white in color, consistent for O. clavigerum.

mtDNA RFLPs.—For HadII and MspI restrictions combined, the number of individual phenotypes was as great as the number of isolates investigated; that is each isolate had its own mitochondrial RFLP pattern. Examples of HadII and MspI restrictions are
FIG. 19. UPGMA cluster analysis of mtDNA RFLP data. Isolates are designated as morpho-species: *L. pyrinum* (underline), *O. clavigerum* (plain), and *L. terebrantis* (bold).

presented in Figs. 17 and 18, respectively. The UPGMA dendrogram developed from combined datasets for *HaeIII* and *MspI* restrictions resolved three major clusters (Fig. 19). The *L. pyrinum* isolates formed one cluster, while a second cluster included all *O. clavigerum* (DJ) isolates, as well as some isolates of *O. clavigerum* (DP). The third cluster included all isolates of *L. terebrantis*, as well as several isolates of *O. clavigerum* (DP).

When *HaeIII* and *MspI* restrictions were analyzed separately, isolates clustered into the same three groups, with the single exception of isolate C813. This isolate grouped with the main *O. clavigerum* cluster in the *HaeIII* dendrogram, but with *L. terebrantis* in the *MspI* dendrogram.

*nDNA fingerprinting.*—Fewer phenotypes were expressed in the nDNA fingerprints compared with the number of phenotypes observed with mtDNA RFLPs. The nDNA fingerprint of the four groups of fungi probed with the (CAC)$_5$ oligonucleotide is shown in Fig. 20.

The UPGMA dendrogram developed from (CAC)$_5$ fingerprints resolved four clusters (Fig. 21). In this case, however, *L. pyrinum* and all isolates of *O. clavigerum* except C813 clearly were delineated in separate clusters. In this dendrogram, isolate C813 again resolved with *L. terebrantis*. C25, an isolate of *L. terebrantis*, resolved with *O. clavigerum*. Other *L. terebrantis* isolates grouped into two clusters separate from *L. pyrinum* and *O. clavigerum*.

**DISCUSSION**

Based on morphology, ecology, mtDNA RFLPs and nDNA fingerprints, *L. pyrinum*, *L. terebrantis*, and *O. clavigerum* appear to be very closely related and might have diverged only recently from a common ancestor. There was no evidence of cryptic species or
physiologic race formation among isolates of *O. clavigerum* associated with *D. ponderosae* and *D. jeffreyi*.

The mtDNA RFLPs and nDNA fingerprints provided additional evidence that *L. pyrinum* is a good species and not a morphologic variant of *O. clavigerum*. However, evidence for the separation of *L. terebrantis* and *O. clavigerum* was not so strong. Most isolates of *L. terebrantis* and *O. clavigerum* clustered in agreement with the morphology consistent for their respective species in the dendrograms produced from mtDNA RFLPs and nDNA fingerprints. In each case, however, inconsistencies were seen. A possible explanation for these inconsistencies is that the two fungi actually comprise a single species that exhibits morphological variation linked to its ecology (mycangial versus nonmycangial). One the other hand, the two fungi might be distinct species that recently have diverged but, because of lineage sorting, are not clearly separable using these genetic markers.

*Leptographium terebrantis* is loosely associated with bark beetle species in several genera (Harrington 1988), while *O. clavigerum* has been found only with *D. ponderosae* (Whitney and Farris 1970) and the closely related *D. jeffreyi* (Six and Paine 1997) and *L. pyrinum* only with *D. adjunctus* (Six and Paine 1996). Furthermore, the unusually long septate and clavate-shaped conidia and large spreading conidiophores of *O. clavigerum* might be derived characters related to ambrosial feeding by the associated beetles. Young adults are known to feed on dense sporogenous fungal growth in pupal chambers for several weeks before emergence from the natal host tree. In addition to the large conidia and spreading conidiophores, *O. clavigerum* produces smaller and less elaborately branched conidiophores and smaller conidia (Tsuneda and Hiratsuka 1984) that are morphologically very similar to those produced by *L. terebrantis*. Thus, while *O. clavigerum* may have retained the conidium and conidiophore morphology of *L. terebrantis*, it also produces a conidium and conidiophore type especially suitable for ambrosial feeding by young adult beetles. We can speculate that *L. terebrantis* might be the more primitive of the three species and that *O. clavigerum* and *L. pyrinum* might well have diverged from *L. terebrantis* when they developed close mycangial associations with specific bark beetles.

Microscopic examination of the conidia and conidiophores of the isolates studied for genetic markers showed three morphological groups that generally showed agreement with the clustering based on nDNA fingerprinting. That is those isolates that produced short, truncate conidia were in the *L. pyrinum* cluster; those that produced long, clavate conidia, often with one or two septations, tended to group in the *O. clavigerum* cluster. Further, the broad, spreading fascicles of conidiophores typical of *O. clavigerum* were seen in isolates of the *O. clavigerum* cluster but not in isolate in the *L. terebrantis* cluster. The two notable exceptions are isolates C25 and C813. The former isolate originally was identified as *L. terebrantis* in Harrington and Cobb (1983) but was found to produce clavate conidia in a later study (Zambino and Harrington 1992), in which it was referred to as *O. clavigerum*. Our more recent examinations of this strain revealed no clavate conidia, but the broad, spreading conidiophores typical of *O. clavigerum* were seen. The insect associate of C25 is not known, but C25 was isolated from *Pinus contorta*, which is a host of *L. terebrantis* and *O. clavigerum* and of the bark beetles, *D. valens* and *D. ponderosae*, that vector these fungi. Placement of C25 in the *O. clavigerum* cluster based on nDNA fingerprinting would suggest that C25 is *O. clavigerum*, but the culture has deteriorated and no longer produced the clavate conidial state, a deterioration that has been noted in other cultures of *O. clavigerum* (Tsuneda and Hiratsuka 1984). The other exceptional isolate is C813, which continues to produce large (55–65 μm), clavate conidia and broad, spreading conidiophores. Thus, C813 is morphologically *O. clavigerum*, though it was isolated from *D. valens* (rather than *D. ponderosae*)
and it clusters with *L. terebrantis* based on nDNA fingerprints. Isolate C813 might be an intermediate in an ongoing process of speciation. It also might be that these two species are not genetically isolated, which also might be suggested by the mtDNA data.

A possible explanation for the resolution of five *O. clavigerum* (DP) associates in the *L. terebrantis* cluster in the dendrogram developed from the mtDNA RFLPs is that after the divergence of *L. terebrantis* and *O. clavigerum*, there has been horizontal transfer of mtDNA, but not nDNA, between the two species.

The horizontal transfer of mtDNA in fungi remains poorly understood, and the mechanisms involved are unknown. The occurrence of such transfers of mtDNA has been suggested in other fungi infecting trees (Brasier et al. 1993, Harrington et al. 1998). It is interesting to note that no isolates of *O. clavigerum* (DJ) showed evidence of possessing mtDNA polymorphisms typical of *L. terebrantis*. This might be due to a lack of contact between *O. clavigerum* (DJ) and *L. terebrantis* in *P. jeffreyi*. *D. valens*, a vector of *L. terebrantis*, can be found commonly in pines colo-
nized by *D. ponderosae* and *O. clavigerum* (DP), and *L. terebrantis* might interact in such trees. *D. valens* also attacks the bases of *P. jeffreyi* attacked by *D. jeffreyi*; however, it is not known how well *L. terebrantis* is able to colonize tissues of this tree. *P. jeffreyi* is quite different chemically than other pines and limits the growth of some ophiostomatoid fungi (Paine and Hanlon 1994, Six and Paine 1998). If growth of *L. terebrantis* is poor in *P. jeffreyi*, *Ophiostoma clavigerum* (DJ) might interface only rarely, or not at all, with this fungus.

On the other hand, the patterns we observed might suggest incomplete lineage sorting accompanied by limited morphological divergence, which can indicate recent or incomplete speciation events (Flowers and Folz 2001). However, this explanation still does not clarify the inconsistencies found using the nDNA probe (CAC)-

About half of the described *Leptographium* species have *Ophiostoma* telemorphs, but the others, including *L. pyrinum* and *L. terebrantis*, are known only by their anamorphs (Harrington 1988, Jacobs and Wingfield 2001) and may be strictly asexual. The mode of reproduction (asexual or sexual) should not affect variation or polymorphism of mtDNA. However, variation and polymorphism of nDNA is predicted to be lower in asexual species than in sexual species. In this study, *Leptographium* species possessed as high or higher nuclear genetic variation than did the sexually reproducing *Ophiostoma clavigerum*. *Ophiostoma clavigerum* possessed the lowest genetic variation and was the least polymorphic of all fungi studied. These results concur with results of a study assaying genetic variation in *O. clavigerum* (DJ) using isozymes, which revealed little genetic variation and polymorphism in this fungus (Six and Paine 1999b).

The low genetic variability in *O. clavigerum* relative to the asexual *Leptographium* species may indicate that sexual reproduction in this species is uncommon. We have not observed the sexual state of *O. clavigerum* in nature, nor have we been able to produce it in artificial culture despite numerous pairings of isolates in the laboratory (Six and Paine 1997, T. C. Harrington unpubl, D. L. Six unpubl). The only known observations of ascocarata for *O. clavigerum* were reported in a pair of related studies by Robinson (1962) and Robinson-Jeffrey and Davidson (1968) in which the authors observed nameless pycnidia (unusual for *Ophiostoma* species) in sapwood and, more rarely, in culture.

The genus *Graphiocladiella* was erected for *Leptographium*-type species with individual conidiophores to those clustered into a synnema-like group (Upadhay 1981). However, in all other respects these fungi appear to be *Leptographium* species (Harrington et al 2001), and *O. clavigerum* is clearly closely related to other *Leptographium* species. Thus we propose to transfer this anamorph to *Leptographium*.


**LITERATURE CITED**


